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**Probability of Alamethicin Conductance States Varies
with Nonlamellar Tendency of Bilayer Phospholipids**

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ABSTRACT

With few exceptions (1-3), membrane lipids are usually regarded as a kind of filler or passive solvent for membrane proteins. Yet, cells exquisitely control membrane composition. Many phospholipids found in plasma membrane bilayers favor packing into inverted hexagonal bulk phases (4-6). It was suggested that the strain of forcing such lipids into a bilayer may affect membrane protein function, such as the operation of trans-membrane channels (7-10, 6). To investigate this, we have inserted the peptide alamethicin into bilayer membranes composed of lipids of empirically determined inverted hexagonal phase "spontaneous radii" R_o , which will have expectably different degrees of strain when forced into bilayer form. We observe a correlation between measured R_o and the relative probabilities of different conductance states. States of higher conductance are more probable in Dioleoyl Phosphatidyl Ethanolamine (DOPE), the lipid of highest curvature, $1/R_o$, than in Dioleoyl Phosphatidyl Choline (DOPC), the lipid of lowest curvature.

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INTRODUCTION

Two poorly understood issues in membrane biology are the mechanism of membrane protein interaction with the lipid bilayer and the constraints upon lipid composition in cell membranes. These two subjects are linked because chemically nonspecific physical parameters which are controlled by membrane composition are known to affect membrane function (e.g. see review (11) by McElhaney) and are therefore likely to limit the variation of lipids allowed in the membrane of a given organelle. Physical parameters such as membrane surface charge, bilayer thickness, and the gel vs. liquid crystalline state of the lipid chains are known to affect protein function but, in general, do not act via a chemically specific protein binding site. There is no reason to believe that we understand, or even recognize, all the physical parameters which are important to membrane function.

The observation of hitherto unrecognized nonspecific physical parameters which affect protein function are important for at least three reasons: (1) They provide clues about the spectrum of lipids in each membrane; (2) They help elucidate general membrane protein mechanisms; and (3) They highlight additional variables which must be controlled in membrane protein reconstitution experiments. It has been suggested that factors relating to the proclivity of certain lipids to form nonlamellar phases, such as the lipid

monolayer spontaneous curvature, may be important physical parameters for membrane and protein function (4, 8-10, 12-15). Transport measurements performed on vesicles have shown the enhancement of protein activity by addition of Hii-forming lipids (16). The purpose of this report is to describe experiments in the DOPC/DOPE system which demonstrate that, indeed, the conductance states of alamethicin reconstituted into planar bilayer are sensitive to lipid type in a way that correlates with the lipid spontaneous curvature.

MATERIALS AND METHODS

Three phospholipids, DOPC, DOPE, and Dioleoyl Phosphatidyl-N-Methyl Ethanolamine (DOPE-Me) and their mixtures were used to make planar Montal-Mueller bilayer membranes (17). DOPC and DOPE were chosen because the spontaneous curvature (8) of the mixture increases monotonically with the DOPC/DOPE ratio (18).

Lipids (Avanti Polar Lipids, Alabaster, AL) were determined to be at least 99% pure by thin layer chromatography. Unsaturated lipids are known to break down slowly on exposure to air. To examine the sensitivity of the data to lipid peroxidation, control experiments were performed in which monooleoyl PC and oleic acid, probable breakdown products, were intentionally added in the amount

of 1/2, 1, and 2% by weight. To within experimental accuracy, these added impurities caused no change in the relative probability or conductance of channel states.

Membranes were formed on a 0.1 mm opening in a 15 micron thick Teflon partition. Hexadecane in n-pentane (1:10) was used for aperture pre-treatment. No change in the observed effects vs. lipid composition were seen when squalane replaced hexadecane as the solvent used to form the bilayer. After bilayer formation in symmetric bathing solutions, alamethicin was added from ethanolic stock solutions to only one side of the bilayer. Approximately 1 microliter of 10^{-5} Molar alamethicin was added to the cis side chamber containing about 1 ml of bathing solution. Analytical grade NaCl (Mallinckrodt, Inc. Paris, KY) was used to prepare the 1.0 M aqueous bathing solutions buffered at pH 6.2 by MES (Calbiochem Corp., La Jolla, CA). All measurements were taken at room temperature of 23 C.

The original experiments utilized a purified form of alamethicin (19). Additional experiments were performed with Sigma alamethicin (St. Louis MO; Cat. No. A4665) which contains at least 3 distinct fractions of alamethicins. We obtained quantitatively equivalent results with this mixed peptide. In particular, we found the same jump (discussed later) in relative probabilities between DOPC and DOPE membranes.

Conductance measurements were made using the 3902 headstage input amplifier of a 3900 Integrating Patch Clamp

System (Dagan Corp., Minneapolis, MN). Current fluctuations were recorded on a digital magnetic tape recorder Unitrade/Toshiba DX-900 (DAS-900) (Unitrade, Philadelphia, PA) operated in pulse code modulation mode. Recorded data were analyzed using a 80386/387 33 MHz computer (Gateway 2000, North Sioux City, SD) with a 12 bit A/D converter board operated at a 3 kHz sampling frequency. Single channel statistics were determined after signal frequency filtering by a 8-pole Bessel filter (Frequency Devices, Haverhill, MA) with a 5 kHz corner frequency. The validity of choice of sampling/filtering frequencies was verified by noise measurements performed on single- and multi-channel recordings. Obtained spectra showed roll-off frequencies of 30-100 Hz, giving no indications of faster processes. It should be mentioned here that the noise of the open channel has high-frequency contributions (20) but their intensity is many orders of magnitude less than the noise generated by switching of the alamethicin channel between different conductance levels.

When dispersions of many phospholipids in excess water are mixed with sufficient amounts of alkanes such as dodecane or tetradecane, they form H_{ii} phases (9, 21, 22). The radii of the water cores of these H_{ii} phases are an empirical measure of the nonlamellar tendency of the lipid mixture. Specifically, the values of R_o correlate with the temperature of the lamellar-nonlamellar phase transitions in the absence of alkane. The physical basis for this measure

and its relationship to phase behavior have been described in detail (23, 7, 12, 18) and reviewed by Gruner (9).

Figure 1 shows the basis vector lengths of Hii phases of the lipids DOPC, DOPE, and DOPE-Me measured via techniques and apparatus described by Kirk and Gruner (21). As explained below, these curves may be used to determine the long spacing of any mixture of the three lipids over the given temperature range. From this information, one can determine the ratios of DOPC, DOPE, and DOPE-Me taken singly, pairwise, or all three together, needed to achieve any given value of R_0 between the extremes exhibited by pure DOPC and DOPE.

The thickness of the lipid monolayer of the Hii phase is ca. 16 Å, hence the remaining water cylinder radius is $R_w = d/2 - 16 \text{ Å}$ and its curvature is $C = 1/R_w \text{ Å}^{-1}$. For succinctness in correlating channel probability and spontaneous curvature, we have assumed that the physical radius of curvature of the Hii tube lipid-water interface may be treated as a monotonic measure of the energetics of bending the lipid monolayer, i.e., that $1/R_w$ may be taken as a "working spontaneous curvature." Although strictly speaking, this radius is equivalent to the spontaneous radius of curvature only of thin membranes (18, 24), such considerations do not affect the essential conclusions of this report. The curvatures reported below are $1/R_w$.

We have found (data not shown) that curvatures of mixed-lipid Hii phases vary as the mole-fraction-average of

the pure-lipid curvatures at the same temperature. For example, if the mole fraction, x , of DOPE in a DOPE + DOPC mixture is $x = [\text{DOPE}]/[\text{DOPE} + \text{DOPC}]$, then the working spontaneous curvature of the mixture, C_{MIX} , is related to the curvature of the pure components, C_{DOPE} and C_{DOPC} , by

$$C_{\text{MIX}} = x \cdot C_{\text{DOPE}} + (1-x) \cdot C_{\text{DOPC}}.$$

This relation allows one to predict the x-ray spacing of the mixture to generally better than an Angstrom. It also allows one to choose different combinations of the three lipids to achieve a given value of the working spontaneous curvature.

RESULTS

Alamethicin, a twenty amino acid peptide of relatively rigid, rod-like structure (25), was incorporated into Montal-Mueller bilayer membranes (blm's) formed from mixtures of three different phospholipids, DOPC, DOPE, and DOPE-Me. At low concentrations, alamethicin exhibits a voltage-dependent ion channel. The conductance of a single ion channel may be quantitatively monitored as a function of time by measuring current across a voltage-clamped macroscopic bilayer.

Bilayers were always clamped with a voltage of 130mV, where the cis side was positive. The single channels we analyzed were the first channels to appear in the membrane upon incorporation of a small amount of alamethicin.

Analysis stopped upon the observance of multiple channels with significant probability of being simultaneously open. Single channels suitable for analysis were recorded for periods up to 45 minutes, long enough to establish that the channel's characteristics were not changing over time. This was checked for each level's probability distribution by comparing results from different segments of the same recording.

Different conductance levels of a single channel were readily distinguished from a possible superposition of independent single channels (a) because observed conductance levels were not simple sums of a fundamental conductance and (b) because probabilities of the higher conductance states were not products of the probabilities of the level-1 state. Also, the density of channel bursts in our recordings was sparse enough to render improbable a significant contribution from a superposition of different single channels.

Typical voltage-dependent single-channel bursts, Figure 2, showed the stepwise access to adjacent conductance states that has been postulated to occur either by the aggregation of monomers or by the opening of portions of existing structures (26). There were clear behavioral distinctions between channels in bilayers of different lipids. Significantly shorter bursts occur in DOPC bilayers than in DOPE, but DOPE bilayers required ten times the solution concentration to form channels.

Even with the striking difference in burst duration and expression of higher level ("4", "5") states, the conductance of each level ("1", "2", "3") did not vary with lipid composition. In other words, single-channel conductances were independent of phospholipid composition. This implies that lipid composition does not alter the essential functional structure of a conductance state. Current bursts always appeared and disappeared through the lowest conducting "0-level" state (following the notation of Eisenberg et al (27, 28)).

However, at a given temperature and applied voltage, when alamethicin single ion channels were observed in bilayers of different lipid compositions, there was a clear difference in the relative probability of the channel being in the n th state, i.e. the ratio of time spent at level n to time in level 1. In blm's of DOPE, which spontaneously forms inverted hexagonal phases in bulk in excess water, 4th and 5th level conductance states were frequently observed; these states were rarely seen in blm's of DOPC, a phospholipid which normally forms lamellar phases in bulk dispersions. DOPE also significantly increased the relative probability of finding the channel in the 2nd and 3rd level states. For example (Figure 3), in pure DOPE the state-3 relative probabilities were more than 50 times those in pure DOPC. Relative probability ratios varied monotonically with lipid ratio.

Given the insensitivity of the magnitude of conductance levels to lipid type, the variation in the relative probability of each level probably reflects changes in relative free energies of the peptide embedded in different host lipids. We conjecture that these energies may be correlated with inherent nonlamellar strain in the blm due to the enforced planar geometry.

The relative probabilities of channel states in DOPE-Me were the same as those in DOPC/DOPE mixtures with the same water core radius. In this sense, channel probabilities can indeed be said to correlate with the lipids' spontaneous tendency to curve to a given Hii-phase radius. Conversely, peptide incorporation appears to change bulk lipid packing. For example, a 1 Mole % concentration of alamethicin in lamellar phase 3:1 DOPE/DOPC mixture produces an isotropic signal as seen by NMR (K. Gawrisch, personal communication) and a complex nonlamellar phase behavior as verified by x-ray diffraction (S.Keller, unpublished observations).

The need for ten-fold higher solution concentrations of alamethicin to obtain single channel currents with DOPE vs. DOPC under the same voltage and solution conditions could indicate differences only in peptide partition between bilayer and solution. However, if lipid sensitivity were due only to differences in partition coefficient, i.e., if solution activities had to compensate to yield the same alamethicin monomer concentration in different membranes, then there would be no change in the channel burst pattern.

Our results demonstrate that the relative probabilities of appearance of higher conductance states change significantly with membrane lipid composition. This suggests that the energetics of channel formation from membrane-bound alamethicin monomers is lipid-dependent.

One might be tempted to argue that the change in the channel pattern described in our report can be explained by higher membrane-bound alamethicin monomer concentration in DOPE bilayers. Indeed, according to Baumann and Mueller (29) "as the concentration of the monomer inside the membrane is increased, either by the addition of more alamethicin or by the applied potential, the average lifetime of the individual n-mers must, for theoretical reasons, increase approximately as the nth power of concentration". However, even if this were the case, then, according to our data, formation of a single channel in DOPE would require a higher monomer concentration, showing lipid-dependent increase in free energy of the channel.

The previous alamethicin literature does not provide definite conclusions about dependence of alamethicin channel level expression on lipid composition. Using several lipids, including glycerol mono-oleate and phosphatidylethanolamines, Gordon and Haydon (30) argued that the probabilities of the conducting levels of the channel are scarcely affected by the membrane composition. Their probability data were obtained from experiments at different transmembrane voltages that could affect

probability distribution and, therefore, make comparison difficult.

Different results, probably depending on the choice of lipid, have been reported for the voltage dependence of the single-channel level probabilities distribution. Hall (31) and Eisenberg et al (27) found nearly no voltage dependence of level distributions. Mueller (32) reported that the most probable channel level changes by one level for every 60mV; and Boheim (33) found a change in one level for every 40-50 mV. To escape this uncertainty we compared channel expression in different lipids at the same transmembrane voltage of 130mV.

Several control experiments were performed to negate the possibility that the observed effects were due to systematic artifacts of the experiments. As explained in "Materials and Methods" the effect is not affected by common lipid impurities or by changing the solvent from hexadecane to squalane. Moreover, the change in relative probability that we observe is not merely due to a change in bilayer thickness between DOPE and DOPC. Trials utilizing 16-carbon chain PE and PC's, whose bilayer thicknesses differ from DOPE and DOPC, produced a change in relative probability at least 10 times smaller than would be expected if our effect were caused only by a change in bilayer thickness.

Alamethicin was chosen as a sufficiently simple peptide to test for correlations between spontaneous curvature and channel expression. Other membrane/protein systems with Hii-

prone lipids can be expected to show similar sensitivities. For example, we observed the expected monotonic change in relative probabilities with $1/R_0$ for alamethicin in bilayers of glycerolmonooleate (GMO), which favors H_{ii} phase, mixed with DOPE or DOPC. However, on the basis of spontaneous curvature, we expected alamethicin in GMO to act as in DOPE; in fact, it acts more as it does in DOPC bilayers. Given the great difference between GMO and phospholipids, alamethicin behavior probably differs qualitatively in the two kinds of lipids (26).

Although many factors other than spontaneous curvature are likely to influence or to determine protein activity, the strains inherent in lipid packing must be recognized in any realistic analysis of proteins in cell membranes.

ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (Grant No. GM32614), the Department of Energy (Grant No. DE-FG02-87ER60522), and the Office of Naval Research (Contract No. N00014-90-J-1702 to S.M.G. and No. N00014-91-F-0201 to V.A.P.). S.L.K. was generously supported by a Liposome Co. Fellowship. We thank Olaf Andersen for critical reading of an earlier version of this article.

FIGURE CAPTIONS

FIGURE 1 Data for water cylinder center-to-center spacing (d , hexagonal basis vector) vs. temperature, for determination of R_0 , the radius of the inverted hexagonal (Hii) phase water core. Triangles - DOPE; filled circles - DOPE-Me; open circles - DOPC.

FIGURE 2 Typical conductance bursts for a single alamethicin channel in Dioleoyl Phosphatidyl Choline (DOPC, left) and Dioleoyl Phosphatidyl Ethanolamine (DOPE, right) bilayers at 130 mV applied trans-membrane potential. Note difference in channel expression but equality of conductance levels 1, 2, and 3.

FIGURE 3 The relative probability of conductance levels for a single alamethicin channel varies greatly with lipid composition and spontaneous curvature. Open symbols stand for the relative probability of Level 2 to Level 1; filled symbols show the relative probability of Level 3 to Level 1. Circles - single partition, DOPC/DOPE membranes; squares - single partition, DOPC/DOPE/DOPE-Me membranes; upward triangles - multiple partitions (a new Teflon partition was used for every lipid mixture), DOPE/DOPC membranes; downward triangles - multiple partitions, DOPC/DOPE/DOPE-Me membranes.

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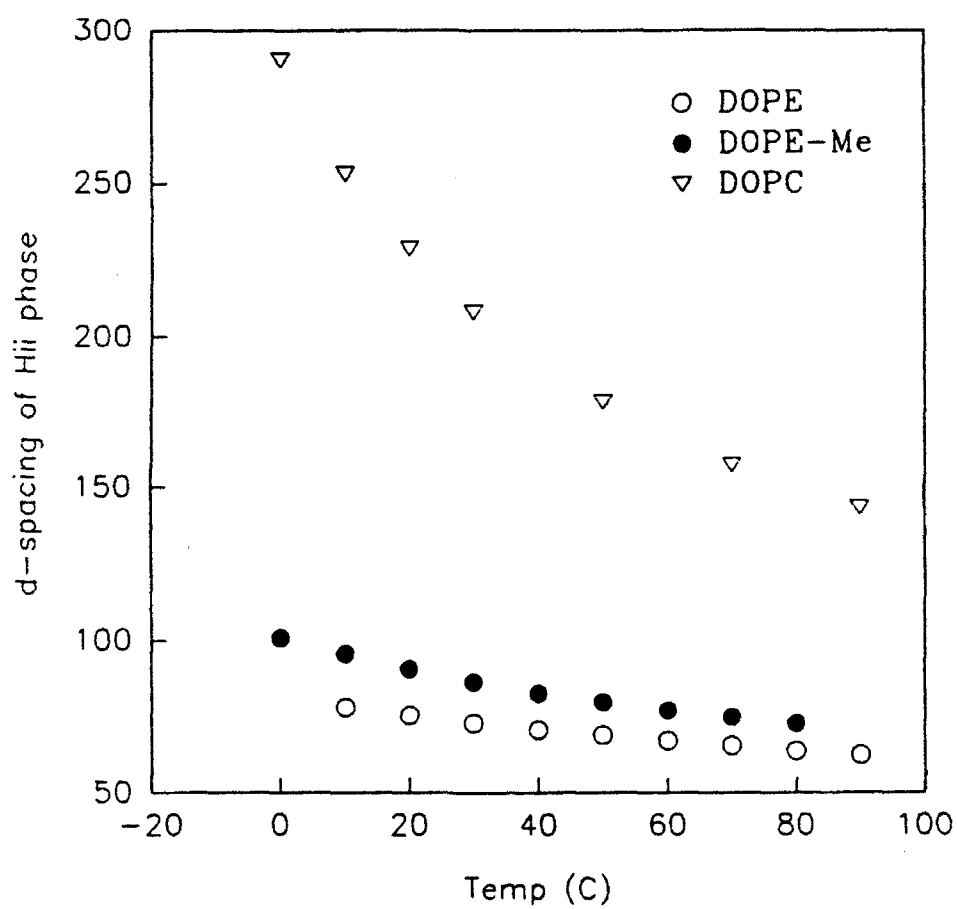
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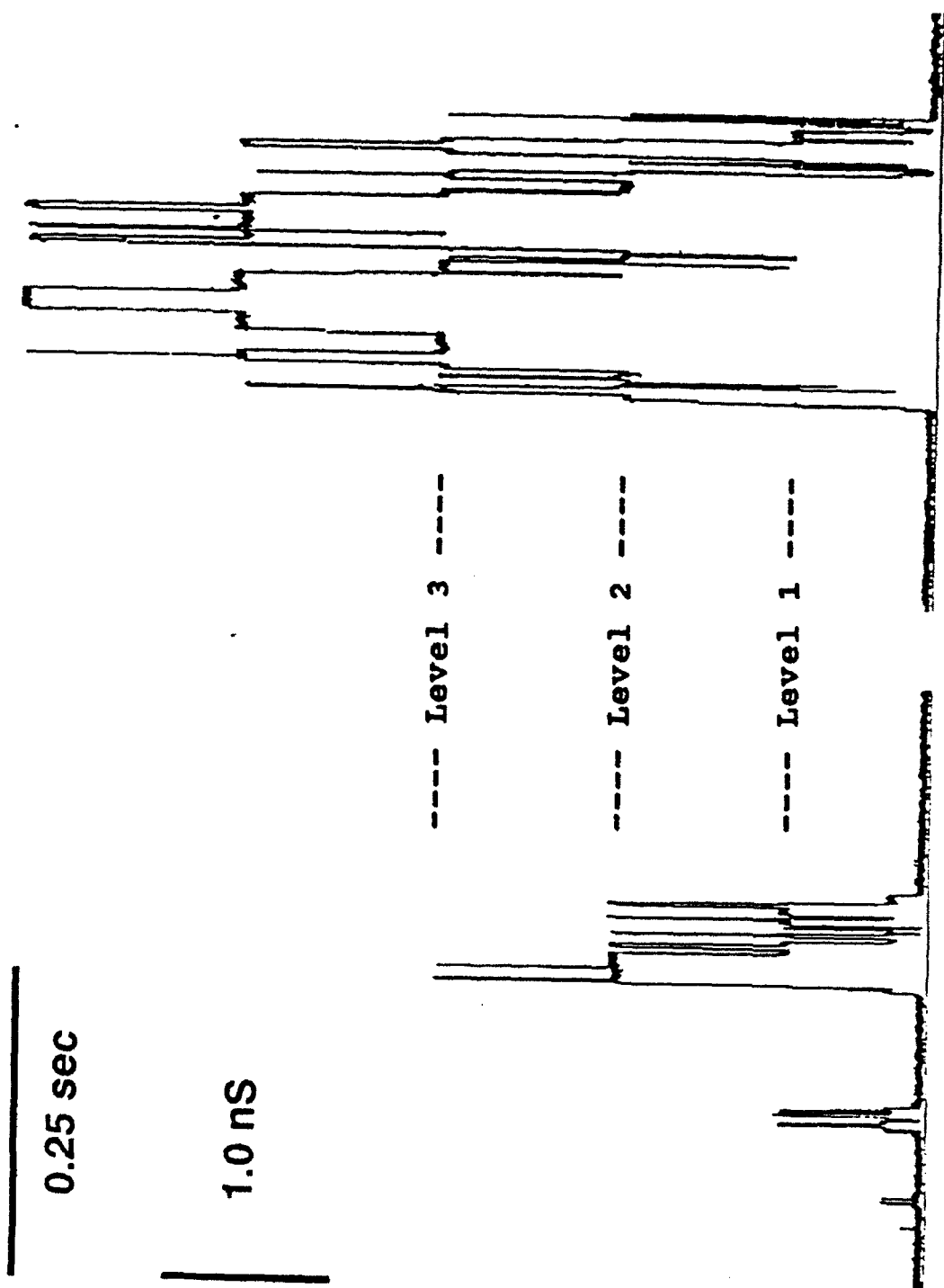
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